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Role of cold shock proteins (Csp) for growth of Listeria monocytogenes under cold and osmotic stress conditions

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Role of cold shock proteins (Csp) for growth of *Listeria monocytogenes* under cold and osmotic stress conditions

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1. Summary

The gram-positive bacterium *Listeria monocytogenes* is a food-borne pathogen of both public health and food safety significance. It possesses three small, highly homologous protein members of the cold shock protein (Csp) family. We used gene expression analysis and a set of mutants with single, double and triple deletion of the *csp* genes to evaluate roles of CspA, CspB and CspD in cold and osmotic (NaCl) stress adaptation responses of *L. monocytogenes*. All three Csps are dispensable for growth at optimal temperature (37°C). These proteins are, however, required for efficient cold and osmotic stress tolerance of this bacterium. The hierarchy of their functional importance differs depending on the environmental stress conditions: CspA>CspD>CspB in response to cold stress versus CspD>CspA/CspB in response to NaCl salt osmotic stress. The fact that Csps are promoting *L. monocytogenes* adaptation against both cold and NaCl stress has significant implications in view of practical food microbial control measures. The combined or sequential exposure of *L. monocytogenes* cells to these two stresses in food environments might inadvertently induce cross protection responses.

Keywords: *L. monocytogenes*, Stress response, Cold stress, NaCl stress

2. Introduction

Listeria monocytogenes is a gram-positive food-borne pathogen of public health concern as well as a food safety challenge. This pathogen primarily encountered through contaminated ready to eat (RTE) foods, infects high-risk individuals such as immunocompromized adults, pregnant women and neonates, and leads to listeriosis, a rare but highly invasive disease with severe clinical signs and relatively high mortality (20-30%) (32, 34). The ubiquity of these organisms in nature and in a wide range of food related environments means that they are a considerable microbial control challenge in food production (9). Moreover, *L. monocytogenes* organisms efficiently adapt and sometimes proliferate despite exposure to low temperatures, low pH and elevated salt (NaCl) concentrations, conditions used in preserving RTE food products (10, 24, 36). Improvement of food safety measures taken against this pathogen will depend on further insights gained into molecular cell response mechanisms underlying the various stress resistance phenotypes properties displayed by these organisms.

The cold shock protein (Csps) family consists of small, highly conserved and structurally-related nucleic acid binding proteins, that presumably have important roles in regulation of various microbial physiological processes (8). These proteins are widely distributed amongst prokaryotes including *L. monocytogenes*, and are often encoded through differentially regulated multiple gene families per organism (16, 29, 38). Csps are thought to serve as nucleic acid chaperones, that bind RNA and DNA, and thus may facilitate the control of processes such as replication, transcription and translation within bacterial cells (8). To date, the multiple homolog nature and functional variability of Csps is mostly derived from studies conducted in *Escherichia*

coli and *Bacillus subtilis*. Nine Csps (CspA-CspI) are found in *E. coli*, and four (CspA, CspB, CspG and CspI) have been linked to modulation of cold adaptation functions (12, 23, 26, 38). The CspC and CspE proteins have been implicated in chromosomal condensation and cell division, and in the regulation of RpoS and UspA stress response proteins (2, 17, 19, 30, 43). Furthermore, the CspD protein of this organism has been suggested to regulate nutrient and stationary phase stress adaptation responses (42). In *B. subtilis*, three Csps (CspA, CspB and CspD) have been described, which have been associated with regulation of both normal growth, as well as cold and stationary phase stress adaptation responses (14, 39). There is, however, limited knowledge regarding the function of Csps found in various psychrotolerant food-borne pathogens (25, 27, 40). An improved understanding of the role of these proteins in normal growth and stress adaptation of *L. monocytogenes* is of particular interest given the challenges these organisms pose to food safety. Three Csp (CspA, CspB and CspD) family proteins are found within the sequenced genomes of *L. monocytogenes*, but their functions are not yet understood (11). The present study was therefore conducted to gain further insights into Csp involvement in normal growth and stress adaptation responses of this bacterium. To achieve this, *csp* gene expression patterns and growth phenotypes of *csp* gene family deletion mutants were evaluated in response to optimal temperature (37°C), cold stress (4 and 10°C) and elevated NaCl salt concentration conditions.

3. Materials and Methods

3.1. Bacteria strains, plasmids and general techniques

The wild type and mutant bacterial strains, as well as the different plasmid vectors used in this study are listed in Table 1. The clinical reference strain *L. monocytogenes* EGDe (11) and its derivatives carrying various *csp* gene family deletion mutants, were used. The *E. coli* host for all plasmid construction and propagation steps was the XL-1 Blue strain (3). The plasmids pKSV7 (32) and pPL2 (21) were employed for genetic disruption and complementation, respectively. Bacterial genomic DNA was isolated with the DNeasy blood and tissue kit (Qiagen). Plasmids were prepared using the Plasmid Midi kit (Qiagen). PCR amplification was performed using the FastStart High Fidelity PCR System (Roche Molecular Diagnostics GmbH, Penzburg, Germany). Restriction enzymes, T4 DNA ligase, and Taq DNA polymerase were used in accordance with protocols of the supplier (Roche). PCR products and DNA restriction fragments were cut and purified from agarose gels using the Mini Elute gel extraction kit (Qiagen). Oligonucleotides were all ordered from Microsynth AG (Balgach, Switzerland). DNA sequencing was also performed at Microsynth AG. Bacterial strains were grown using brain-heart infusion (BHI) or Luria-Bertan (LB) (Difco laboratories) or chemically defined minimal media (DM; (31)). Osmolarity in DMS (2.2% NaCl) and BHI plus 3% NaCl media were adjusted by addition of NaCl to the desired levels.

3.2. Genetic deletion and complementation

A set of *csp* gene deletion mutants was created in the EGDe strain background. The splicing-by-overlap extension (SOE) PCR protocol was used for

non-polar deletions of the *csp* gene family (18). The *ΔcspA*, *ΔcspB* and *ΔcspD* constructs were created by PCR amplification of EGDe DNA templates using the various SOE primers listed in Table 2. The SOE PCR products were, thereafter, cloned into the pKSV7 plasmid using the incorporated restriction enzyme sites. The pKSV7-Δ*cspA*, pKSV7-Δ*cspB* and pKSV7-Δ*cspD* plasmid constructs were confirmed by DNA sequencing. The deletion of the targeted genes through homologous recombination and allelic exchange was facilitated following previously described protocols with some modifications (4). Briefly, *L. monocytogenes* cells were transformed with the plasmid DNA constructs as previously described (28). Transformants were selected on LB plates in presence of chloramphenicol (10µg/ml) after 48-72 hrs of incubation at 30°C. Integration mutants were enriched for 3-6 generations at 42°C in 10 ml BHI broth plus chloramphenicol (10µg/mL). The integrated strains were grown on BHI agar plates in presence of chloramphenicol (10µg/ml) after 24-48 hrs incubation at 42°C. Colonies of integrants were confirmed after colony PCR analysis with a primer combination that bind defined regions in the pKSV7 plasmid and EGDe genomic DNA, respectively. Plasmid excision was facilitated through growth of the integrants in BHI broth for 3-50 generations. During this period, samples were drawn at every third generation and plated out on to BHI agar plates. The colonies were thereafter plated onto BHI plates with and without antibiotic to identify chloramphenicol sensitive colonies in which the plasmid had been excised. PCR analysis and subsequent DNA sequencing of the targeted regions confirmed deletion mutants. The Δ*cspA*, Δ*cspB* and Δ*cspD* single deletion mutant strains were generated initially. These strains were subsequently used in a second round of mutagenesis to target a second *csp* gene to create the Δ*cspAB*, Δ*cspAD* and Δ*cspBD* double deletion mutants. Finally, the *cspA* gene was targeted in the Δ*cspBD*

strain, generating the Δ*cspABD* triple deletion mutant strain. Defective phenotypes of Δ*cspA* and Δ*cspD* strains were complemented using a pPL2 plasmid based system as described (21). DNA fragments consisting of the complete *cspA* and *cspD* genes as well as their entire upstream sequence between them and the preceding gene, were amplified from the EGDe DNA templates and subsequently cloned between the *PstI* and *SmaI* restriction sites of the pPL2 vector to create the pPL2-*cspA* and pPL2-*cspD* complementation plasmids. Upstream sequences included the predicted promoter elements and ribosome binding sites preceding these two genes. These constructs were confirmed by DNA sequencing and were subsequently used to transform the Δ*cspA* and Δ*cspD* strains, respectively. Colonies of transformants obtained on LB agar plates in presence of chloramphenicol (10 µg/ml) were analyzed for proper integration of pPL2-*cspA* and pPL2-*cspD* constructs into the PSA prophage integration site by PCR analysis using the previously described NC16/PL95 primer set (21).

3.3. Growth conditions

Stationary phase inoculums (10^9 CFU/ml) of wild type and mutant strains were prepared by growth of single colonies in 10 ml BHI, overnight (16-18 hrs) in a shaking incubator (220 rpm) at 37°C. The stationary phase inoculums were 10-fold serially diluted to 10^5 CFU/ml in 0.85% NaCl-peptone. Thereafter, aliquots of 100 µl were used for inoculation of 10 ml BHI, DM or DMS resulting in 10^3 CFU/ml starter cultures. Growth evaluation in BHI and DM at different temperatures involved preparation of three duplicate sets from each strain, which were subsequently incubated at 37, 10 and 4°C respectively, without shaking. For growth experiments in DMS (DM plus 2.2 % NaCl), 10 ml duplicate cultures were inoculated and similarly

incubated at 37°C. The growth kinetics in each sample was monitored by standard colony counting at defined time intervals. The results presented in this study depict means and standard deviations of three independent experimental runs.

3.4. Cold and NaCl stress adaptation experiments

For cold stress exposure, 10 ml BHI stationary phase cultures of the wild type EGDe strain, were prepared by overnight growth at 37°C. The stationary phase cultures were centrifuged (4000g for 5 min), and the pellets were resuspended in 10 ml fresh BHI and divided into two 5 ml aliquots, which were habituated to 4°C and 37°C, respectively, by 2 hrs of incubation. Thereafter, 1.5 ml samples were drawn and total RNA templates were isolated as outlined below. For NaCl stress exposure, stationary phase inoculums, prepared as described above, were used to inoculate 10 ml of BHI or BHI plus 3% NaCl at 10^3 CFU/ml. These cultures were then incubated without shaking at 37°C for 10 (BHI) and 15 (BHI-NaCl) hrs, respectively to reach the late log phase, which was confirmed by colony counting in each case. At this point, 1.5 ml aliquots were drawn and directly processed for total RNA as outlined below or frozen at -20°C until processed at a later date.

3.5. Total RNA extraction

The RNA extraction procedure employed combined rapid mechanical lysis and column based RNA purification. The 1.5 ml aliquots from each culture were centrifuged at 4°C (cold adapted cells) or room temperature (non-cold adapted cells) for 5 minutes at 4000g. The supernatants were discarded and pellets were resuspended in 0.5 ml lysis buffer provided in the RNeasy Plus Mini Kit (Qiagen). The mixtures were transferred onto the lysing bead matrix in MagNA lyser tubes (Roche Molecular

Diagnostics GmbH, Penzburg, Germany). Bacterial cells were mechanically disrupted using the MagNA Lyser Instrument (6500 rpm for 60 secs). Total RNA was purified from the lysates on RNA binding columns according to the RNeasy Plus Mini Kit protocol and included two DNA removal steps. The cell lysates passed through a genomic DNA removal column and an on-column DNAse I digestion step was performed. Total RNA was eluted using 30 µl of RNAse-free water and the yield was determined by measuring absorption at 260 nm using the Nanodrop ND1000 (Nanodrop instruments, Delaware, USA). RNA template integrity was further analyzed under UV light following denaturing agarose gel electrophoresis and ethidium bromide staining.

3.6. Reverse transcription

Reverse transcription (RT) was performed using the Quantitect Reverse Transcription Kit (QIAGEN). 300 ng of total RNA from each sample were converted into cDNA in 20 µl reactions. Similar amounts of total RNA from each sample were also subjected to the cDNA synthesis reaction without the reverse transcriptase enzyme as controls. These were subsequently used as minus RT controls to assess potential residual DNA contamination in total RNA templates.

3.7. Real-time PCR and quantification of gene expression

Primers used in this study are listed in Table 2. The real-time PCR reactions were performed in the Light Cycler 480 instrument (Roche Molecular Diagnostics, Rotkreuz, Switzerland). The reactions were performed in a final reaction volume of 10 µl: 2.5 µL (3.75 ng) cDNA (1:10 dilution of cDNA generated as described above); 2.5 µL (0.4 µM) of gene specific forward and reverse primer mix; 5 µL of

2XLightCycler^{R} 480 SYBR Green I master mix. The real-time PCR run protocol comprised of: preincubation (4 min at 95°C); 40 amplification cycles (10s at 95°C; 20s at 56°C; 20s at 72°C; 5s at 80°C with a single fluorescent measurement); melting curve (65-97°C at 2.2°C/s plus continuous fluorescent measurement). Primers and reaction conditions were optimized for specificity and target amplification with efficiencies of 90 to 100%. Transcript levels were determined using the Light Cycler 480 Relative Quantification Software (Roche Molecular Diagnostics). Initially a reference gene validation under the different experimental conditions was performed as previously described (37), and the *16S rRNA* transcripts were established as the most suitable internal control reference gene for relative mRNA quantification in our study. Based on this, were quantified *csp* gene transcripts relative to *16S rRNA* transcript level of the same sample.

3.8. Statistical analysis

Statistical analysis was performed using the Stat View 4.02 (Abacus Concepts Inc., Berkeley, California, USA) program. The bacterial colony counts were converted into log CFU/ml followed by calculation of the mean and standard deviations. The statistical significance of differences in the bacterial colony counts between the wild type and the *csp* mutants were evaluated using one-way analysis of variance (ANOVA) at $\alpha = 0.05$ significance level. The comparative data derived from quantitative gene expression analysis were analyzed using the Students t-test and differences with P values <0.05 were considered to be statistically significant.

3.9. Bioinformatics

The Vector NTI Advance 10.3 program (Invitrogen) was used for DNA and

Protein sequence manipulations. Protein motif search and hairpin predictions were performed using the DNASIS MaxVersion 2.6 (Miraibio, San Francisco, USA). Sequence homologies and protein sequence alignments were investigated using the Blastn and Blastp (NCBI) and the multiple sequence alignment with hierarchical clustering programs (7).

4. Results

4.1. Comparative nucleotide and amino acid sequence analysis of *L. monocytogenes* Csps

Nucleotide sequence comparison of Csps in the EGDe strain revealed high levels of sequence similarity with identities ranging from 77 to 84% (data not shown). All the *csp* ORFs in this organism are preceded by relatively long stretches of 5'-untranslated leader regions (5'UTR) ranging from 198 bp (*cspA*) to 363 bp (*cspD*). A situation which is similar to what has previously been described for *csp* genes of other bacteria species (1, 35). These long 5'UTR regions were predicted to be rich in RNA hairpin loop secondary structures (DNASIS MaxVersion 2.6 program, Miraibio, San Francisco, USA), which might have implications for the regulation of *csp* gene expression in this organism. The amino acid sequence comparison matrix of *L. monocytogenes* EGDe Csps, and homologs of *E. coli* ($CspA_{Ec}$) and *B. subtilis* ($CspB_{Bs}$) are presented in Table 3. As shown *L. monocytogenes* CspA, CspB and CspD also share high levels of amino acid sequence identity (67-73%). These proteins are also closely related to the *B. subtilis* (72-80%) and *E. coli* (58-62%) homologs. In fact *L. monocytogenes* CspA and CspB are more closely related to *B. subtilis* $CspB_{Bs}$ (74-80%) than to the other Csps of this organism. An amino acid sequence alignment

further highlights general sequence conservation and the canonical nucleic acid binding motifs, RNP-1 and RNP-2 (Fig 1). Although some amino acid substitutions are found within the RNP-1 and RNP-2 motifs these are conserved. As shown a tyrosine to phenylalanine substitution occurs in *L. monocytogenes* CspB RNP-1 and an isoleucine to valine substitution in *L. monocytogenes* CspB and CspD RNP-2 motifs compared to the motifs of *E. coli* and *B. subtilis* homologs.

4.2. Cold stress associated induction of *csp* gene expression

The effect of *L. monocytogenes* cold adaptation on the expression pattern of its *csp* gene family was examined. To achieve this, stationary phase *L. monocytogenes* EGDe cells originally grown at 37°C, were adapted to cold stress conditions by incubation for 2hrs at 4°C. As a control, an equal aliquot of the same EGDe culture was adapted to optimal temperature conditions by similar incubation for 2 hrs at 37°C. Total RNA isolated from these samples was used to determine the level of *csp* gene family (*cspA*, *cspB* and *cspD*) transcripts in quantitative RT-PCR assays. A summary of the relative *csp* gene expression ratios at 4 and 37°C, as well as cold stress dependent fold-induction are presented in Table 4. As shown, there is low constitutive expression of *cspA*, *cspB* and *cspD* transcripts in EGDe cells held at 37°C, but all *csp* gene transcripts are significantly induced ($P<0.05$), to varying extents, in cold adapted EGDe cells at 4°C. Under these conditions mean cold stress dependent fold inductions of 23-fold, 4.5-fold and 7.4-fold were observed for *cspA*, *cpsB* and *cspD* transcripts, respectively.

4.3. NaCl stress dependent induction of *cspA* and *cspD* gene transcripts

Next, the influence of EGDe strain growth under NaCl stress on *csp* gene family expression was investigated. For this purpose, late log phase EGDe cells grown in BHI plus 3% NaCl and controls that had been similarly grown in regular BHI were compared. The csp gene expression results from these studies are presented in Table 5. As shown both *cspA* and *cspD* transcripts were also significantly enhanced ($P<0.05$) in NaCl stress adapted EGDe cells compared to the control of regular BHI grown EGDe cells. In contrast, there was no statistically significant increase associated with *cspB* ($P>0.05$) transcripts in NaCl stress adapted EGDe cells (Table 5). The mean NaCl stress dependent fold inductions achieved were 2.8-fold and 4.4-fold for *cspA* and *cspD* transcripts, respectively.

4.4. Creation of *cspA* deletion mutants in *L. monocytogenes* EGDe

To gain more insights into the phenotypic contributions of Csps to *L. monocytogenes* growth under different conditions, various deletion mutants were created in the EGDe strain (Table 1). The deletions of the targeted Csps were all performed in-frame and verified through PCR and DNA sequence analysis of the targeted DNA regions (data not shown). Furthermore, quantitative RT-PCR assays with gene specific primer pairs confirmed that transcripts corresponding to targeted *cspA* genes, were also absent in the relevant mutant strains (data not shown). The phenotypic restoration in Δ*cspA* and Δ*cspD* strains was accomplished using pPL2-*cspA* and pPL2-*cspD* plasmid constructs. This process restored deleted *cspA* and *cspD* genes by integration into the PSA prophage *attB* site of the *L. monocytogenes* EGDe chromosome in respective mutant strains.

4.5. Csps are required for efficient *L. monocytogenes* growth under cold stress

The growth kinetics of the wild type and the various *cspA* deletion mutant strains are summarized in Table 6. Representative growth curves depicting the growth of the wild type and selected *cspA* deletion mutants are presented in Figure 2. As shown individual *cspA, cspB* or *cspD* gene deletions resulted in no detectable growth phenotype differences from the parent wild type strain at 37°C in rich (BHI) and minimal (DM) nutrient conditions (Figs. 2A and 2B). Similarly, double deletions as well as triple deletion of all known *csp* genes, produced no detectable growth phenotype defects at 37°C as summarized by Table 6. Meanwhile the growth analysis at 4°C showed that *cspB* deletion also had no influence, whilst deletion of *cspA* or *cspD* impairs *L. monocytogenes* growth under cold stress (Figs. 2C and 2D). In fact, the Δ*cspA* strain completely failed to grow at 4°C and 10°C in BHI and DM. In support of a causal link, the cold growth phenotype of the Δ*cspA* strain was successfully restored by a pPL2-*cspA* complementation (Fig. 2E). The cold growth phenotype in the Δ*cspD* strain was only significantly poor ($P<0.05$) compared to the wild type at 4°C but not at 10°C (Figs. 2C and 2D and Table 6). The cold sensitive phenotype of this mutant was primarily observed in minimal defined nutrient conditions of DM. But it was less pronounced within the rich complex nutrient background in BHI (see Figs. 2C and 2D). Similarly growth analysis of double (Δ*cspAB*, Δ*cspAD*, Δ*cspBD*) and triple (Δ*cspABD*) Csp deletion strains further confirmed that *cspA* or *cspD* gene deletions compromises the cold growth phenotypes of *L. monocytogenes* EGDe (Table 6). Whilst *cspB* gene functions seemed dispensable as long as *cspA* and *cspD* genes were retained in this bacterium. This hypothesis was confirmed by cold growth analysis of a Δ*cspDB* mutant strain in DM at 4°C (Fig. 2F). As shown growth in this mutant strain is further compromised by *cspB* deletion, when

compared to growths of the Δ*cspD* or Δ*cspB* strains (Fig. 2F). Thus loss of *cspB* gene functions, combined with a *cspD* deletion, also leads to impaired cold growth of EGDe cells, suggesting some minimal CspB protein contribution to cold adaptation functions of this organism.

4.6. *L. monocytogenes* Csps contribute to efficient cellular growth under NaCl stress

The growth responses of the wild type and various *cspA* deletion mutants were also investigated in 2.2 % NaCl salt supplemented minimal defined media (DMS). The loss of *cspD* gene function significantly compromises NaCl stress tolerance capacity of EGDe cells (Fig. 4A). The growth of the Δ*cspD* in DMS was significantly slower than the wild type strain. This was despite the fact that both strains grew similarly in regular DM (Fig. 2B). Phenotypic defects of Δ*cspD* growth in DMS were partly restored through pPL2-*cspD* based genetic complementation (Fig. 3A). On the other hand individual *cspA* and *cspB* gene deletions did not influence EGDe growth phenotypes in DMS (Fig. 3B and Table 7). A double deletion of these two genes, however, significantly reduced growth in DMS (Fig. 3B). The loss of *cspD* gene function in *cspA/D* (Δ*cspAD*) and *cspB/D* (Δ*cspBD*) double deletion mutants also confirmed to impair growth phenotypes of EGDe under NaCl stress conditions of DMS (Table 7). A *cspA*/*B*/*D* triple deletion mutant also leads to complete growth failure of EGDe cells in DMS, indicating that at least one Csp must be retained for *L. monocytogenes* growth at high NaCl salt concentrations.

5. Discussion

Csp family proteins have previously been linked to regulation of both normal growth, and stress adaptation processes against cold and nutrient starvation as well as promotion of survival stationary phase stage cells in some organisms (13-15, 41, 42). In cold adapted *L. monocytogenes* cells, the induction of Csp-like proteins, as well as *cspA* gene transcripts, were previously documented (5, 40). The current study was conducted in order to expand this knowledge and to explore the Csps contribution in other stress responses of this organism.

In contrast to *B. subtilis*, where at least one *csp* gene is essential for viability (14), we found that none of the three *csp* genes is essential for *L. monocytogenes* viability. In fact, a mutant carrying deletion in all three *csp* genes could be generated without any discernable defects in the growth phenotype at 37°C, even under the defined minimal nutrient conditions of DM. It thus appears that functions encoded by the *L. monocytogenes* Csps are not critical requirement for viability or efficient growth under optimal temperature conditions. On the contrary a hierarchy (*cspA*>*cspD*>*cspB*) was established in *csp* gene importance to cold adaptation and *L. monocytogenes* growth at low temperatures (4 and 10°C). The deletion of *cspA* gene completely abolishes cold growth, and *cspD* deletion leads to significantly reduced cold (4°C) growth efficiency of this organism. The functions of the *cspB* gene on the other hand are largely dispensable. There were no cold growth defects associated with the deletion of this gene as long as *cpsA* or *cspD* genes were retained.

The potential role of Csps in osmotic stress adaptation so far stems from the fact that expression of the *cspC* gene in *B. bronchiseptica*, is significantly induced following exposure of this organism to 2M NaCl (35). We show here that direct

growth of *L. monocytogenes* EGDe under NaCl stress in BHI-NaCl leads to significant induction of *cspA* and *cspD* gene expression. Although the overall NaCl stress associated *csp* fold inductions appeared lower compared to those in cold adapted cells. The induction trend observed of *cspD>cspA* was consistent with the subsequent trend observed in the stress sensitivity (Δ*cspD* >Δ*cspA* or Δ*cspB*) of the *csp* gene deletion mutants. In fact, single deletions of *cspA* or *cspB* gave no discernable NaCl stress growth phenotypes in DMS cultures. The Δ*cspAB* double deletion mutant was however also significantly impaired during growth under similar NaCl stress conditions. It also seems that at least one *csp* gene is required for *L. monocytogenes* growth at higher NaCl concentrations. A Δ*cspABD* triple deletion mutant completely failed to grow under NaCl stress conditions in DMS cultures.

In this study, we have presented both gene expression and stress growth phenotypic evaluation data suggesting that *L. monocytogenes* Csps are functionally required for efficient cold and osmotic stress adaptation responses in this bacterium. Although the precise nature of Csp involvement in such stress adaptation mechanisms still remain to be investigated. Key cold stress challenges stem include negatively super coiled DNA and stabilized RNA secondary structures. These impair cellular replication, transcription and translation processes. Therefore, increased Csp synthesis and activity at low temperatures provides DNA and RNA chaperone functions (8,29), that are needed in cold exposed *L. monocytogenes* cells to help resolve these nucleic acid structural hurdles. The Csp functional contributions to NaCl osmotic stress adaptation in *L. monocytogenes* are not yet clear. One possibility is that Csp chaperones might also promote the increased production of sodium ion extrusion transporter proteins through their effects in facilitating transcription and translation processes. This might enhance protection of *L. monocytogenes* cells from NaCl

toxicity through increased extrusion of intracellular sodium ions. Alternatively, as observed in eukaryotic cells, high cellular NaCl concentration might lead to increased levels of cellular DNA damage in *L. monocytogenes* cells (20). It is thus plausible that Csps through their DNA chaperone activity facilitate repair of NaCl stress damaged DNA and promote *L. monocytogenes* under NaCl associated osmotic stress.

The fact that Csps seem to promote *L. monocytogenes* adaptation against both cold and NaCl stress also has significant implications in view of practical food microbial control measures. The combined or sequential exposure of *L. monocytogenes* cells to these two stresses in food environments might inadvertently induce cross protection responses. Cold stress induced by low temperature may inadvertently cross protect cells against NaCl stress due to induction of *cspA*, *cspD* and *cspB* gene expression. Conversely, exposure to higher NaCl salt concentrations in some foods before cooling can also adapt cells to grow at low temperatures due to induction of *cspA* and *cspD* gene functions in response to NaCl stress. A similar phenomenon was recently described for the spoilage bacterium *Shewanella putrefaciens* (22) and in an *lmo 1078* gene transposon deletion mutant of *L. monocytogenes* (6). A mutant in this gene, which encodes a putative UDP-glucose phosphorylase enzyme, also exhibited cold and NaCl salt stress sensitivity.

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7. Tables

Table 1. Bacterial strains and plasmids

	Genotype	Genetic deletion	References
Strain			
EDGe	Wild type (1/2a)	None	(11)
Δ*cspA*	Δ*cspA*	153 bp in-frame *cspA* deletion	This study
Δ*cspB*	Δ*cspB*	147 bp in-frame *cspB* deletion	This study
Δ*cspD*	Δ*cspD*	147 bp in-frame *cspD* deletion	This study
Δ*cspAB*	Δ*cspAB*	*cspA* and *cspB* in-frame deletions	This study
Δ*cspAD*	Δ*cspAD*	*cspA* and *cspD* in-frame deletions	This study
Δ*cspABD*	Δ*cspABD*	*cspA*, *cspB* and *cspD* in-frame deletions	This study
Plasmids			
pKSV7			(33)
pKSV7- Δ*cspA*			This study
pKSV7- Δ*cspB*			This study
pKSV7- Δ*cspD*			This study
pPL2			(21)
pPL2-*cspA*			This study
pPL2-*cspD*			This study

Table 2. Oligonucleotide primers[1] used in this study[2]

Primer	Sequence
SOE-P1-CspA-A[a]	GGAATTCCGGAAAACAAACAAGTGACGCT
SOE-P1-CspA-B	TGCTTGAGGTCCGCGACCTTGTTCCATGTTCATGTTCCT
SOE-P1-CspA-C[e]	AGG*AACATGGAACAAGGTCGCGGACCTCAAGCAG*CT
SOE-P1-CspA-D[b]	ACGCGTCGACCACCATCACCGATTATCGGAATA
SOE-P2-CspB-A[c]	TCCCCCGGGGCGTCCGGCAAGTGTGAATTGGTTAGTGAGCGAG
SOE-P2-CspB-B	TGCTTGTGGGCCACGAACTGTACCTGTTTGCATATTTCACAA
SOE-P2-CspB-C[e]	*AACAGGTACAGTTCGTGGCCCACAAGCA*GAAAAAG
SOE-P2-CspB-D[c]	TCCCCCGGG CGCACTGGAATACAAGCTGGGACTATGCCCGCTTT
SOE-P3-CspD-A[c]	TCCCCCGGGCCACCATTTTTGTTACAGAGGAAGGA
SOE-P3-CspD-B	CAATGCAAAAT GGAAAGTACGCGGCGCTCAAG CAG
SOE-P3-CspD-C[e]	*CTGCTTGAGCGCCGCGTACTTTCCCATTTTGCATTG*AAATAAATCC
SOE-P3-CspD-D[c]	TCCCCCGGGCGCACTGGAATACAAGCTGGGACTATGCCCGCTTT
CspA-comp-P1[d]	AAAACTGCAG CTGATTTAATCGCACTTAGAGAAAATTAATCA
CspA-comp-P2[c]	TCCCCCGGG TTACGCTTTTTGAACGTTAGCTGCTT
CspA-fw	AACATGGAACAAGGTACAG*
CspA-rv	GTTGGCCTTCTTCAACG*
CspB-fw	CAAACAGGTACAGTTAAATGGTTTA*
CspB-rv	ACGATTTCAAATTCAACGCTTTGA*
CspD-fw	TACGGTTTTATCGAATCAGAC*
CspD-rv	ACGTTAGCTGCTTGAG*
16S rRNA-fw	CTTCCGCAATGGACGAAAGT*
16S rRNA-rv	CTCATCGTTTACGGCGTG*

[1] Oligonucleotides were synthesized at Microsynth AG (Balgach, Switzerland);

[2]Primers were designed using the LC probe and primer design software (Roche Molecular Diagnostics GmBH, Penzburg, Germany)

[a]The *EcoRI* restriction site incorporated in this primer to facilitate cloning is underlined

[b]The *SalI* restriction site incorporated in this primer to facilitate cloning is underlined

[c]The *SmaI* restriction site incorporated in this primer to facilitate cloning is underlined

[d]The *PstI* restriction site incorporated in this primer to facilitate cloning is underlined

[e]The complementary overhangs in SOE-P1-CspA-B, SOE-P1-CspB-B and SOE-P1-CspD-B primers are in italics

*Quantitative real-time RT-PCR primers

Table 3. Amino acid sequence comparison matrix of *L. monocytogenes* Csps, *E. coli* CspA and *B. subtilis* CspB.

	Sequence identitity (%)				
	$CspA_{Lm}$	$CspB_{Lm}$	$CspD_{Lm}$	$CspA_{Ec}$	$CspB_{Bs}$
[1]$CspA_{Lm}$	100%				
$CspB_{Lm}$	73%	100%			
$CspD_{Lm}$	73%	67%	100%		
[2] $CspA_{Ec}$	62%	59%	58%	100%	
[3]$CspB_{Bs}$	74%	80%	72%	61%	100%

[1]Lm, *L. monocytogenes*, [2]Ec, *E. coli* and [3]Bs, *B. subtilis*

Table 4. Relative *csp* gene expression induction of cold stress adaptated stationary phase EGDe cells[1].

Gene	Relative gene expression ratios		Fold induction[2] 4°C vs 37°C
	37°C	4°C	
cspA	0.02±0.01	**0.46±0.20**	23
cspB	0.37±0.20	**1.48±0.30**	4
cspD	0.05±0.04	**0.37±0.10**	7.4

[1]*csp* gene transcripts are expressed relative to the level of 16S *rRNA* reference gene transcripts. [2]Fold induction in *csp* gene expression ratios in cold adapted at 4°C relative to the optimal (37°C) temperature adapted controls.

Table 5. Relative *csp* gene expression induction of BHI-NaCl stress adaptated log phase EGDe cells[1]

Gene	Relative gene expression ratios		Fold induction[2] BHI-NaCl vs BHI
	BHI	BHI-3% NaCl	
cspA	0.06±0.01	**0.17±0.02**	2.8
cspB	0.56±0.31	**0.83±0.35**	NS[3]
cspD	0.15±0.01	**0.66±0.32**	4.4

[1]*csp* gene transcripts are expressed relative to the level of 16S *rRNA* reference gene transcripts. [2]Fold induction in *csp* gene expression ratios of BHI-NaCl grown cells relative to the regular BHI grown controls.

[3]Gene expression induction differences between stress and non-stress exposed controls cells were not statistical significant (P>0.05).

Table 6. An overview of *csp* gene deletion mutant growth phenotypes at 37, 10 and 4°C[1]

Strain	BHI			DM		
	37°C	10°C	4°C	37°C	10°C	4°C
Wild type	++++	++++	++++	++++	++++	++++
Δ*cspA*	++++	-[B]	-	++++	-	-
Δ*cspB*	++++	++++	++++	++++	++++	++++
Δ*cspD*	++++	++++	+++	++++	++++	++
Δ*cspAB*	++++	-	-	++++	-	-
Δ*cspAD*	++++	-	-	++++	-	-
Δ*cspBD*	++++	++++	+++	++++	++++	+
Δ*cspABD*	++++	-	-	++++	-	-

[1]Growth phenotype of each mutant strain is expressed relative to the wild type strain.

[A]level of mutant strain growth phenotype relative to the wild type strain; [B]No growth

Table 7. An overview of Csp deletion mutants growth phenotypes in DM and DMS [1]

Strain	DM	DMS
Wild type	++++	++++
Δ*cspA*	++++[A]	++++
Δ*cspB*	++++	++++
Δ*cspD*	++++	++
Δ*cspAB*	++++	++
Δ*cspAD*	++++	++
Δ*cspBD*	++++	++
Δ*cspABD*	++++	-[B]

[1]Growth phenotype of each mutant strain is expressed relative to the wild type strain.

[A]level of mutant strain growth phenotype relative to the wild type strain; [B]No growth

8. Figure legends

FIG. 1. Amino acid sequence alignment of *L. monocytogenes* Csps ($CspA_{Lm}$, $CspB_{Lm}$ and $CspD_{Lm}$) and the *E. coli* ($CspA_{Ec}$) and *B. subtilis* ($CspB_{Bs}$) homologs. The sequences were aligned using the multiple sequence alignment with hierarchical clustering program (6). Dots represent identical amino acids in all the five Csp homologs, and dashes represent the gaps placed in the sequences by the alignment program to optimize the alignment. The highly conserved nucleic acid binding motifs (RNP1 and RNP2) of cold shock domain proteins are highlighted in bold type and underlined.

FIG. 2. Growth of the wild type EGDe and various *csp* gene family deletion mutants of this strain. A-D) Growth kinetics of the wild type, Δ*cspA*, Δ*cspB* and Δ*cspD* strains in BHI and DM at optimal (37°C) and cold stress (4°C) temperatures; E) Growth of wild type, Δ*cspA* and pPL2-*cspA* complemented Δ*cspA* strains at 10°C in BHI; F) Growth kinetics of Δ*cspB*, Δ*cspD* and Δ*cspBD* at 4°C in DM. The results represent means (±standard deviations) from duplicates of three independent experimental runs.

FIG. 3. Growth of the wild type EGDe and various *csp* gene family deletion mutants under NaCl stress in DMS. Growth kinetics in DMS of: A) wild type, Δ*cspD* and pPL2-*cspD* complemented Δ*cspD* strains; B) wild type, Δ*cspA*, Δ*cspB* and Δ*cspAB* strains. The results are means (±standard deviations) from duplicates of three independent experimental runs.

9. Figures

Figure 1

```
                          RNP1          RNP2
LmCspA   -MNMEQGTVKWFNAEKGFGFIERE-NGDDVFVHFSAIQGDGFKSLDEGQAVTFDVEEGQRGPQAANVQKA-
LmCspB   ---.QN.K.....N...Y....SD-G.E.I....T......Y...E.......E.V..N..A.....E...
LmCspD   ---.QT.......S........V.-G...I.......E.E...T.....S.E.EIV........EK.T.L.
BsCspB   ---.LE.K.....S........V.-GQ............E...T.E.....S.EIV..N........T.EA
EccspA   MSGKMT.I......D......TPDDGSK..........N..Y.......K.S.TI.S.AK..A.G..TSL-
```

Figure 2

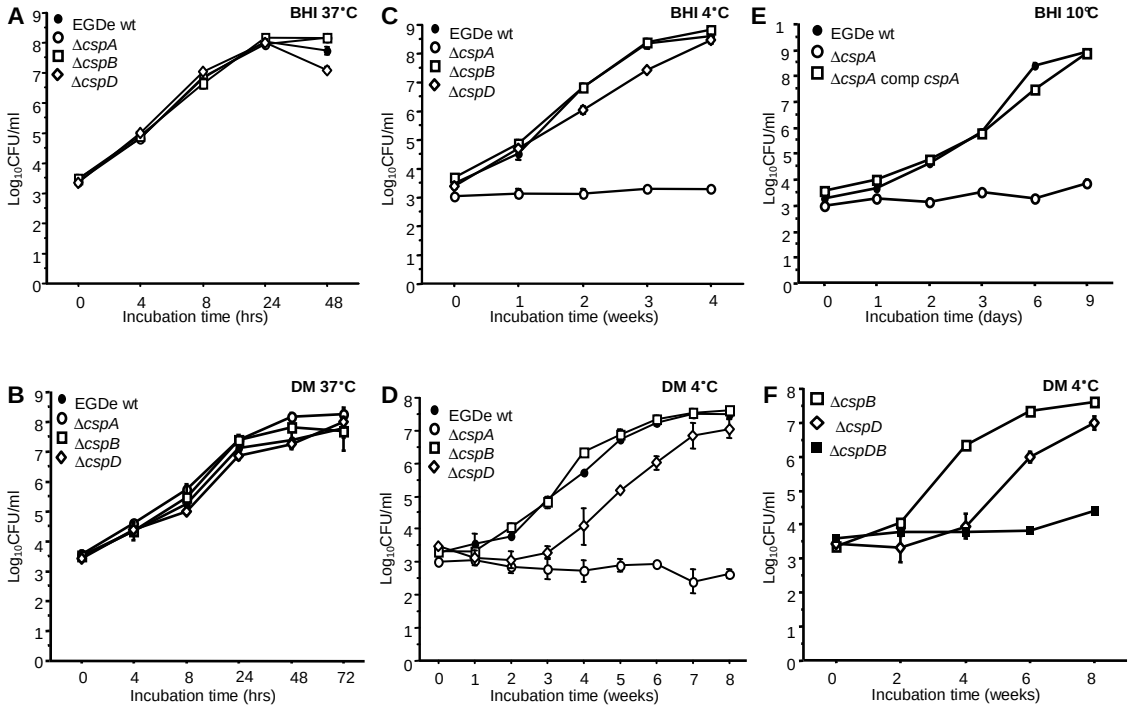
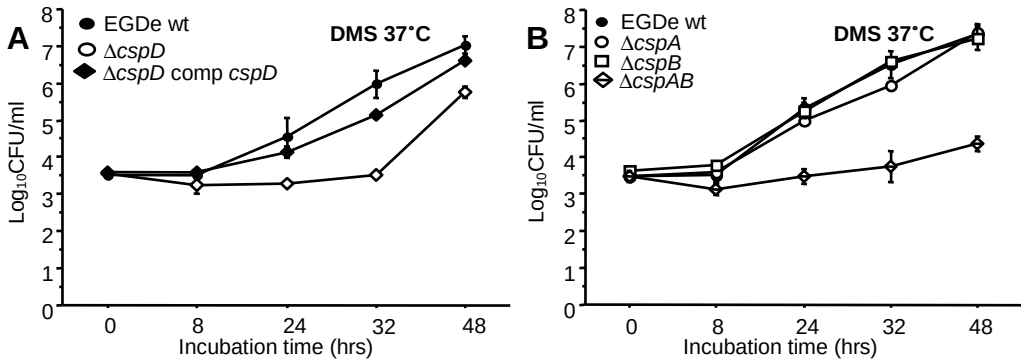


Figure 3


A
EGDe wt
ΔcspD
ΔcspD comp cspD
DMS 37˚C
Log10CFU/ml
Incubation time (hrs)
B
EGDe wt
ΔcspA
ΔcspB
ΔcspAB
DMS 37˚C
Log10CFU/ml
Incubation time (hrs)

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